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I further certify that pursuant to the provisions of Section 38(1) of the Patents Act 1990 a complete specification was filed on 01 February 2000 and it is an associated application to Provisional Application No. PP 8448 and has been allocated No. 26480/00.

WITNESS my hand this
Twenty-third day of September 2002

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P/00/009 28/5/91
Regulation 3.2

ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "A Method For Detecting Methylated Nucleic Acids"

The invention is described in the following statement:

A METHOD FOR DETECTING METHYLATED NUCLEIC ACIDS

FIELD OF INVENTION

The present invention relates to a method for the detecting the presence of methylated nucleic acids. In particular, it relates to a method of diagnosing cancer by detecting the methylation of nucleic acids.

BACKGROUND ART

The methylation of cytosine residues in DNA is currently thought to play an active role in controlling normal cellular development. Various types of studies have demonstrated that a close correlation exists between methylation and transcriptional inactivation. Considerable evidence has established that in vertebrates, inactive genes often contain the modified cytosine residue 5-methylcytosine (mC) followed immediately by a guanosine (G) residue in the DNA sequence. Regions of DNA that are actively engaged in transcription, however, lack 5' methylcytosine residues.

There is now considerable evidence suggesting that alterations in the DNA methylating machinery may play an important role in tumorigenesis and tumour progression. In this respect, focal hypermethylation and generalised genomic demethylation are recognised features of many different types of neoplasms.

Targets for regional hypermethylation are normally unmethylated "CpG islands" located in gene promoter regions. This hypermethylation correlates with transcriptional repression that can serve as an alternative to coding region mutations for inactivation of tumor suppressor genes, including p16, p15, VHL, and E-cad. How general genomic hypomethylation and hypermethylation of some specific regions, in particular, evolve during tumorigenesis is just beginning to be defined. Normally, unmethylated CpG islands appear protected from dense methylation affecting immediate flanking regions. In neoplastic cells this protection

is lost, possibly by chronic exposure to increased DNA-methyltransferase activity and/or disruption of local protective mechanisms.

The hypermethylation of certain genes can also have a role to play in the control of the cell cycle. One such gene is the Myf-3 gene. The Myf-3 gene is normally hypomethylated in non-malignant tissues. Recent studies have indicated that the Myf-3 gene is dramatically hypermethylated in many types of cancer tissues. Therefore, a simple method of detecting hypermethylation of Myf-3 should provide new approaches to detection and diagnosis of some cancer.

Mapping of methylated regions in DNA has relied primarily on Southern Blotting techniques, based on the inability of methylation-sensitive restriction enzymes to cleave sequences that contain one or more methylated cytosine residues. This method is relatively insensitive, requires large amounts of high molecular weight DNA and can only provide information about those cytosine residues found within sequences recognized by methylation-sensitive restriction enzymes. A further disadvantage of the Southern Blotting methods is that the whole procedure requires 7-10 days.

The Polymerase Chain Reaction (PCR) has also been used to detect methylated DNA. In this method, methylation sensitive enzymes are employed to distinguish between methylated and non-methylated DNA. More specifically, PCR primers are designed to span a region of DNA that includes a restriction endonuclease recognition sequence that is sensitive to DNA methylation. If the enzyme recognition sequence is not methylated the DNA is hydrolysed and the PCR target DNA is destroyed. If the DNA is methylated the enzyme does not hydrolyse the target and DNA chain synthesis is achieved. Restriction of unmethylated DNA must be complete, since any uncleaved DNA will be amplified by PCR. This can lead to a false positive result for methylation. A further problem is that this method involves multiple steps.

A third method combines PCR with bisulphite treatment of DNA to convert all unmethylated cytosines to thymine. Methylated cytosine residues are protected from conversion to thymine by bisulphite. PCR primers that are specific for converted or unconverted cytosine residues are used to generate DNA chain synthesis including the cytosine residues under investigation. Usually cloning and sequencing steps are required to assign which cytosine residues are methylated. This method is technically demanding, labour intensive and without cloning amplification products, requires approximately 10% of the alleles to be methylated for detection.

Current methods of detecting DNA methylation are time consuming, expensive and often lack specificity. The present invention seeks to ameliorate these and other problems associated with the prior art.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers including method steps.

DISCLOSURE OF THE INVENTION

The present invention provides a method for detecting methylated nucleic acids comprising the steps of:

1) Contacting a nucleic acid sample suspected of containing methylated nucleotides with an oligonucleotide probe under suitable conditions for nucleic acid hybridization, said probe characterised in that,

   (i) it comprises a first stem labeled with a fluorophore moiety, a loop sequence having a region of nucleotides complementary to at least a region of the nucleic acid sample, which region is susceptible to methylation, and a second stem labeled with a quencher moiety that is

capable of quenching the fluorophore moiety when in close spatial proximity to the fluorophore moiety; and

(ii) the nucleotides forming the first stem are capable of hybridising to the nucleotides forming the second stem when the probe is dissociated from the nucleic acid sample;

2) altering the hybridization conditions such that the oligonucleotide probe dissociates from unmethylated DNA but remains hybridized to methylated DNA; and

3) measuring the change in fluorescence.

When the loop sequence in the probe binds a complementary sequence in a target gene the probe enters an "open conformation" and fluorescence of the donor fluorophore is detectable. When the probe is in a closed (hairpin) conformation, the fluorescence of the donor fluorophore is quenched.

Desirably the loop sequence is complementary to a portion of a nucleic acid sequence that undergoes methylation when a cell transforms from a normal state to a cancerous state. Further the loop sequence is preferably selected such that it is capable of specifically hybridizing with the target sequence, but is unable to form internal structures which favour maintenance of the probe in a "closed configuration" (ie when the two stems hybridise together). In a particularly preferred probe design, the probe is designed to hybridise to a region in a nucleic acid sequence that has a high proportion of CG oligonucleotides that have the potential to be methylated.

In one preferred embodiment, the present invention may be used to detect the presence of methylation of a gene (ie. a gene's transformation from a relatively unmethylated state to a more methylated state). Methylation of various genes has been implicated in the onset or development of cancerous states in various cells. One such gene that is known to undergo such a transformation is the Myf-3 gene.

Other genes which may contain regions of DNA which are normally unmethylated but become hypermethylated in neoplastic cells include parts of the developmental PAX genes and glutathione-S-transferase-Π(pi).

As a gene becomes methylated there is a progressive increase in the melting temperature of that nucleic acid species. Thus the melting temperature between a probe bound to methylated nucleotides as distinct from unmethylated nucleotides is higher. The present invention capitalises on this characteristic to distinguish unmethylated and methylated nucleic acids.

When the probe encounters a target DNA sequence, it forms a hybrid that is longer and more stable than the hybrid formed by the stem sequences. Since nucleic acid double helices are relatively rigid, formation of a probe-target hybrid precludes the simultaneous existence of a hybrid formed by the stem sequences. Thus the probe undergoes a spontaneous conformational change that forces the stem sequences to move away from each other, causing the probe to enter an "open conformation," wherein fluorescence can be detected since the fluorophore is no longer in close proximity to the quencher. Unhybridized probe will not fluoresce.

Detection of methylation according to the invention is achieved by altering the hybridization conditions to facilitate dissociation of the probe from unmethylated DNA while retaining the hybridization bonds between probe and methylated DNA. Such detection is preferably achieved by raising the temperature of the hybridization reaction. As this temperature increases, a point will be reached where the probe melts away from the target sequence. Dissociation of the probe from the target provides the probe with conformation freedom therein enabling the stem structures to hybridise. Hybridization of the stem structures brings the fluorophore and the quencher into a spatial proximity that quenches the fluorescence. By measuring a change in fluorescence in the reaction, it is possible to detect when a probe melts away from its complementary sequence in the gene of interest.

To ensure that the stem structures rehybridise following their dissociation from the target gene, the melting temperature of the stem hybrid should at least be higher than the melting temperature of the loop plus stem sequence when hybridized to its complementary sequence. Preferably the melting temperature of the stem hybrid is greater than the melting temperature of the loop sequence when bound to a methylated nucleic acid sequence which is complementary to the loop sequence. The melting temperature of the probe will depend upon the length of the hybrid stem, the G-C content of the arm and the concentration of the salts in the solution in which it is dissolved. Desirably, the melting temperature of the two stems in the probe is at least 2 degrees greater than the hybridisation temperature of loop plus stem sequence with the target sequence. More preferably, the melting temperature of the two stems in the probe is at least 5 degrees greater than the hybridisation temperature of loop sequence with the target sequence. In one example of the invention the melting temperature of the two stems in the probe is at least 10 degrees greater than the hybridisation temperature of loop sequence with the target sequence.

Where the melting temperature of a particular probe when annealed to a methylated and an unmethylated gene is known, the hybridization conditions may be varied by increasing the hybridization temperature to a temperature which causes the loop sequence to melt away from unmethylated complementary target gene sequence. Because the stem sequences reassociate following separation from unmethylated gene sequence a drop in fluorescence will be observed. The fluorescence of the reaction mixture can then be measured.

Where the melting temperature of a probe bound to a methylated and/or unmethylated nucleic acid sequence is not known or there is uncertainty about the melting temperature, a control reaction is preferably performed in parallel with the test sample. The control reaction should contain a corresponding gene sequence which is either unmethylated or methylated or, if desired, both unmethylated and methylated reactions may be run as two separate controls.

The term "molecular beacon" as used herein refers to a molecule capable of participating in a specific binding reaction and whose fluorescent activity changes when the molecule participates in that binding reaction. The oligonucleotide probe referred to above is for the purposes of this invention a molecular beacon.

A oligonucleotide probe is "hybridizable" to a nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single-stranded form of the probe can anneal to the nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, 1989, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Hybridization requires that the probe and the nucleic acid molecule contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on variables well known in the art. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. The greater the degree of complementary between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., 1989, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., 1989, *supra*, 11.7-11.8). Preferably the loop sequence comprises at least a minimum number of nucleotides to avoid non-specific binding between the probe and its

target nucleic acid sequence. Desirably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; more preferably at least about 15 nucleotides; most preferably the length is at least about 20 nucleotides. In a highly preferred form of the invention the loop sequence is a structure of approximately 18 or 19 bases.

While the loop sequence should contain at least about 10 nucleotides, the stem sequence, may comprise any nucleic acid sequence that ensures hybridization between the two stems when in a free (ie. unbound) state. In this respect, the stems need not be of equal length. If the stems are of different lengths, they must be capable of bringing the fluorophore and the quencher into spatial proximity to quench the fluorescence of the fluorophore when the two stems hybridize to each other. Desirably stem sequences will not hybridise to the probe's target gene and are of a sufficiently short length to avoid non-specific binding between the stem and any other nucleic acid sequence in the reaction mixture. The minimum and maximum lengths of the stems should preferably be about 4 and 8 nucleotides respectively.

Mg^{++} has a powerful stabilizing influence on the stem hybrid. In a particularly preferred embodiment of the invention the hybridization reaction is carried out in the presence of a sufficient concentration of Mg^{++} ions which is capable of facilitating stem hybrid formation. It is preferable that the reaction mixture contains $MgCl_2$ to increase the stability of the hybrid. Preferably the concentration of $MgCl_2$ is 5mM to 10mM.

A further consideration for the length of the probe as a whole will be the need to create a minimum distance between the fluorophore and the quencher moieties used in the probe when in the open conformation to avoid quenching of the fluorophore. This optimal distance will vary with the specific moieties used, and may be easily determined by one of ordinary skill in the art using techniques known in the art. Desirably, the loop sequence is at least twice the length of the stem sequences to ensure that the conformational change occurs upon hybridization and

to ensure that the fluorophore is sufficiently far from the quencher to restore full fluorescence. Preferably the moieties are separated by a distance of up to 35 nucleotides, more preferably from 10-25nucleotides, and still more preferably from 15-20 nucleotides.

To avoid false positives in the method, the amount of probe employed in the reaction is desirably at least equal to or greater than the relative amount of target nucleotide sequence in the sample. Determining the concentration of target nucleotide sequence in the sample may be achieved by any method known in the art.

Oligonucleotide probes used in the invention can be synthesized by a number of approaches, e.g. Ozaki *et al.* (1992) Nucleic Acids Research, 20:5205-5214; Agrawal *et al.* (1990) Nucleic Acids Research, 15:5319-5423 or the like. The oligonucleotide probes of the invention are conveniently synthesized on an automated DNA synthesizer, e.g. an Applied Biosystems, Inc. Foster City, Calif.) model 392 or 394 DNA/RNA Synthesizer, using standard chemistries, such as phosphoramidite chemistry, e.g. disclosed in the following references: Beaucage and Iyer, (1992) Tetrahedron, 48:2223-2311; U.S. Pat. No. 4,980,460; U.S. Pat. No. 4,725, 677; U.S. Pat. Nos. 4,415,732; 4,458,066 and 4,973,679; and the like. Alternative chemistries, e.g. resulting in non-natural backbone groups, such as phosphorothioate, phosphoramidate, and the like, may also be employed provided that the hybridization efficiencies of the resulting oligonucleotides and/or cleavage efficiency of the exonuclease employed are not adversely affected.

The fluorophore and the quencher may be attached to the probe by any means that enables quenching of the fluorophore when in a closed conformation and illumination of the fluorophore in an open conformation. For example the moieties may be attached to the probe by chemical linkers etc. Preferably the fluorophore and or the quencher molecule are attached to the 5' or 3' terminal nucleotide in the probe. Methods for attaching such moieties to a nucleotide probe are known to

those skilled in the field.

A fluorophore is a chemical compound which when excited by exposure to particular wavelengths of light, emits light (ie. fluoresces) at a different wavelength. Fluorophores and quencher molecules participate in fluorescence resonance energy transfer (FRET).

In FRET, energy is passed non-radioactively over a long distance (1 - 10nm) between the flurophore and a quencher molecule. The fluorophore absorbs a photon and transfers this energy non-radioactively to the quencher. When the fluorophore and the quencher are in close proximity and the emission and absorption spectra overlap the energy of the fluorophore is transferred to the quencher without subsequent emission of fluorescence. Preferably, the nature of the fluorophore-quencher pair is such that energy received by the fluorophore is transferred to the quencher and dissipated as heat, rather than being emitted as light. As a result the fluorophore is unable to fluoresce.

Combinations of a fluorophore and an interacting molecule such as a quenching moiety are known as "FRET" pairs. The primary requirement for FRET is that the emission spectrum of the fluorophore overlaps with the absorption spectrum of the quenching molecule. The efficiency of energy transfer decreases proportionately to the sixth power of the distance between the fluorophore and quencher molecules. One of ordinary skill in the art can easily determine, using art-known techniques of spectrophotometry, which fluorophores and which quenchers will make suitable FRET pairs. Molecules that are commonly used in FRET pairs include fluorescein, 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'dichloro-6-carboxyfluoroscein (JOE), rhodamine, 6'carboxyrhodamine (R6G), N,N,N',N'-tetramthyl -6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL), and 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS). Preferably, FAM (which has an emission maximum of 525 nm) is used as a fluorophore for TAMRA, ROX, DABCYL and R6G (all of which have an excitation maximum of 514 nm) in

a FRET pair. Alternative the fluorophore may be EDANS and the quencher may be DABCYL.

The above method has general application to detect any DNA sequence which is methylated in cancerous tissue while hypomethylated in non-cancerous tissue. In one embodiment of the invention the probe is directed against at least a target sequence in the Myf-3 gene. A preferable target sequence of the Myf-3 gene is as follows:

5' GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3'

5' TTA TAC CGC AGG CGG GCG AGC CGC GGG CGC TCG CT - 3'

5' CCG AGA GCC CTG CGG GGC CCG CCC TCC TGC TGG CG - 3'

The above sequence is a particularly preferred target for the probe of the molecular beacon for the following reasons:

i) it contains a high density of CG dinucleotides which have the potential to be methylated;

ii) the intervening sequences include sufficient numbers of the nucleotides A and T in order to make the beacon specific for Myf-3;

iii) the beacons designed to hybridise with this region are unable to form any inappropriate internal structures which may favour maintenance of the beacon in a closed configuration; and

iv) the melting temperature of the two stems of the molecular beacons must be at least 10 degrees greater than the hybridisation temperature of loop component of the molecular beacon and the target sequence of the Myf-3 gene.

In a highly preferred form of the invention the temperature of the hybridization reaction is maintained at 20°C for 20 minutes and increased from 25°C to 45°C at increments of 2° with a 2 minute stabilising time. As the temperature is increased from 50°C to 90°C at increments of 2°C every 5 minutes the fluorescence of the reaction mix is monitored. Still preferably, the temperature is allowed to increase from 50°C to 95°C in 2' increments with each temperature being held for 1 minute.

The present invention also includes reagent kits that include labeled probes according to this invention, together with other reagents for an assay. For example, a kit may include enzymes, primers and buffers for a PCR reaction together with Molecular Beacons for detecting amplified product. For multiplex assays, kits according to this invention will include multiple probes, at least one of which is a probe according to this invention.

BRIEF DESCRIPTION OF FIGURES

The following non-limiting Figures and Examples further describe the present invention. In the drawings:

Figure 1 shows a denaturation profile of various reaction mixtures containing methylated and non-methylated target DNA sequences. As illustrated in the figure (◆) represents that use of molecular beacon B against an excess of target non-methylated Myf-3, (■) represents the use of molecular beacon B against an excess of target methylated Myf-3, (▲) represents molecular beacon A against an excess of target non-methylated Myf-3, (×) represents the use of molecular beacon A against an excess of target methylated Myf-3, (*) represents the use of an equal amount of target against non-methylated Myf-3, and (●) represents molecular beacon A against an equal amount of target methylated Myf-3.

Figure 2 shows the denaturation profile of molecular beacon A with non, 1, 2, 3, 4 and 5, 5-methylated oligonucleotides. As illustrated in the figure (■) represents the use of molecular beacon A against the preferred Myf-3 target with no methylated

cytosines, (◆) represents the use of molecular beacon A against the preferred Myf-3 target with one 5-methylated cytosine, (▲) represents the use of molecular beacon A against the preferred Myf-3 target with two 5-methylated cytosines, (●) represents the use of molecular beacon A against the preferred Myf-3 target with three methylated cytosines, (×) represents the use of molecular beacon A against the preferred Myf-3 target with four methylated cytosines, and (○) represents the use of molecular beacon A against the preferred Myf-3 target with five methylated cytosines.

BEST MODE(S) FOR CARRYING OUT THE INVENTION

The present invention will now be described by way of example only. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the invention, and should not be understood in any way as a restriction on the broad description as set out above. In particular, it will be understood that all temperature ranges and other such variables prescribed in the examples are given as indicative only, and that parameters outside these limits may also provide useful results.

EXAMPLES

Probes

Two molecular beacons with loop sequences complementary to the preferred region of the Myf-3 gene were purchased from the Midland Certified reagent company. Molecular beacon A contained a 18-nucleotide long complementary loop sequence and a 6 nucleotide-stem sequence (see Table 1). Five of the 12 stem nucleotides were also complementary to the target sequence.

Molecular beacon B contained the same 18-nucleotide complementary loop sequence with a 5-nucleotide stem sequence (see Table 1). The probe sequence is one base longer in molecular beacon A than in B with the extra base creating an

extra base pair adjacent to the stem. Four of the 10 stem nucleotides were also complementary to the target sequence.

Target DNA

To investigate the temperature profiles of methylated and nonmethylated DNA using molecular beacons, oligonucleotides of 32 bases representing the preferred region of the Myf-3 gene and complementary to the molecular beacons were purchased from Bresatec. The oligonucleotides contain 7 CG sites which include cytosine residues that are able to be methylated. In the 5M oligonucleotide, cytosines within each of the 7CG dinucleotides were replaced with 5-methylated cytosines. The Non-5M oligo remained unmethylated. All the oligonucleotides and the molecular beacons purchased were HPLC purified.

Table 1: Oligonucleotide sequences

Molecular beacon A	5'-(6FAM)CGA GGC GGG CTG GAC GCG TCG GAG GCC TCG(DABCYL)-3'
Molecular beacon B	5'-(6FAM)CGA GGG GGC TGG ACG CGT CGG AGCCTC G(DABCYL)-3'
Complementary strand A	5'CTC CGA CGC GTC CAG CCC G-3'
Complementary strand B	5'-CTC CGA CGC GTC CAG CCC-3'
5M	5'-GC_mG GC_mG ACT CC_mG AC_mG C_mGT CCA GCC C_mGC_m GCT CC-3'
Non-5M	5'-GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC-3'

C_m = 5 methlyated cytosines
5M = preferred Myf-3 target sequence, 5M methylated, Non-5M unmethylated (see page 12, line 8).
The stem sequences are underlined

Thermal denaturation profiles

Molecular beacons are known to linearize and hybridize with complementary DNA at certain temperatures releasing fluorescence as an indicator of hybridization. In order to determine the effects of changing temperatures on the level of fluorescence, thermal denaturation profiles were carried out with different oligonucleotides and molecular beacons. Three different temperature conditions were investigated and the best denaturation profile was chosen for further study. The fluorescence was monitored during each profile by using a fluorescence reader with a programmed temperature control (PE/ABI 7700 Applied Biosystems).

The first profile included increasing the temperature from 55°C-95°C at 1° steps with each temperature being held for 1 minute. In the second profile, the temperature was maintained at 20°C for 20 minutes and increased from 25°C to 45°C at increments of 2° with a 2 minute stabilising time. The fluorescence was monitored as the temperature was increased from 50°C to 90°C at increments of 2° every 5 minutes. In another profile the same steps were carried out with the fluorescence detected as the temperature was increased at increments of 1° every 5 minutes.

The fluorescence intensity of at each temperature was plotted as a function of temperature on a linear scale from 0-100%. Representative profiles are shown in Fig. 1.

Reaction mix

Melting curve experiments were conducted by adding appropriate concentrations of oligonucleotides to a reaction mixture containing 20mM tris-HCl (pH 8.0), 50mM KCl and 5mM $MgCl_2$. The molecular beacons were added to a final concentration of 2.98μM. The reaction mixtures were made to a final volume of 50 μl with double distilled water. The beacon was added to the reaction mixture at the very last minute, in order to minimise any molecular beacon/target interaction prior to the temperature control.

Hybridization Conditions

In order to determine the hybridization efficiency and the minimum amount of oligonucleotide needed for hybridization, the oligonucleotides were added in equal, limited and excess amounts to the reaction mixture (ie. Equal: 2.98μM, Limited: 1.99μM, 0.99μM, Excess: 5.96μM, 11.92μM respectively).

Determination of the effects of salt concentration

Divalent cations such as Mg^{++} are known to have powerful stabilising effect on the stem hybrids. The effects of different salt concentrations on denaturation profiles of molecular beacon B with strand A and B were investigated. Reaction mixtures containing equal, limited and excess amounts of oligonucleotides were subjected to 10mM, 5mM and 0mM concentrations of $MgCl_2$.

Thermal denaturation profiles with methylated oligonucleotides

Molecular beacon A and B were added to a final concentration of 2.98μM to reaction mixtures containing equal, limited and excess amounts of methylated oligonucleotide, (5M) and non-methylated oligonucleotides, (non-5M) (2.98μM, 1.49μM and 5.98μM respectively). The reaction mixtures were then subjected to the temperature profile: 20°C for 20min, 25°C-35°C in 2° increments each 2min, and 50°C to 90°C at 2° increments every 5min where fluorescence was monitored.

Determination of the Hybridisation Profile

In order to construct a denaturation profile for the molecular beacons with complementary strand A and B, the temperature was allowed to increase from 50°C to 95°C in 2° increments with each temperature being held for 1min. This resulted in a typical sigmoid curve. When the temperature was raised, the fluorescence was

increased. This was due to a conformational change of the molecular beacon from the stem loop structure to a random coil structure.

According to Tyagi et al., Nature 1996 14(3): 303-308 molecular beacons hybridise spontaneously to their targets at room temperature. Therefore the mixture was incubated initially at 20°C for 20 min to allow for effective hybridisation of the target to the molecular beacon. The fluorescence reaches a maximum at approximately 50°C.

It remained high until a certain temperature was reached, where the % fluorescence decreased rapidly or reached 0% fluorescence. This temperature represented the temperature at which the probe-target hybrid melts. For molecular beacon B with complementary strand A and B the hybridisation melt temperature was approximately 74°C-76°C. The hybridisation melting temperature for molecular beacon B was approximately 74°C. In the above incidences the % fluorescence reached 0% after the hybridisation melt temperature was reached. On the other hand % fluorescence of beacon A with strand A dropped around 76°C-78°C but did not reach 0% even at 90°C.

With the above mentioned temperature profile it was possible to measure consistent temperature profiles as well as allowing enough time for molecular beacon-target hybridisation.

The amounts of oligonucleotide needed for hybridisation

In order to determine the minimum amounts of oligonucleotide necessary for hybridisation, molecular beacons A and B were added to reaction mixtures containing equal, limited and excess amounts of oligonucleotide. No distinct hybridisation melt temperature was observed in limited (1.49µM or 0.795µM) oligonucleotide samples with the exception of 1.49µM oligonucleotide sample with molecular beacon B. A hybridisation melt temperature of approximately 66°C with

complementary strand A and approximately 72°C with complementary strand B was observed.

The maximum fluorescence intensity reached with equal amounts of oligonucleotides was lower than the reaction mixtures with excess oligonucleotide. The hybridisation temperature was also elevated in the excess oligonucleotide samples. For example molecular beacon with equal amounts of strand A had a hybridisation temperature of 66°C, while for the same reaction mixture with excess strand had a melt temperature of approximately 74°C. It was generally concluded that for effective molecular beacon-target hybridisation to occur, it is necessary to have the target either in excess or at least in equal amounts.

Effects of salt concentrations on the denaturation profiles

Analysis of different salt concentrations with molecular beacon B with strand A and B indicated that the presence of $MgCl_2$ in the reaction mixture increases the stability of the hybrids as predicted. In both limited and equal amounts of oligonucleotide, a higher intensity of fluorescence was detected in samples with 5mM $MgCl_2$ concentration. But when the salt concentration was increased from 5mM to 10mM only a marginal increase in fluorescence intensity was observed.

Methylation detection

The hybridisation melting temperature observed when molecular beacons A and B were hybridised to the methylated oligonucleotide 5M was higher than the melting temperatures of beacons A and B hybridised to the non-methylated oligonucleotide, Non-5M.

For example, as shown in Fig. 1, molecular beacon A with an equal amount of 5M melted at approximately 80°C while the non-methylated Non-5M melted at approximately 69°C. Molecular beacon A with excess amounts of 5M had a hybridisation melt temperature of approximately 76°C while for the non-5M it was

approximately 71°C. With molecular beacon B and excess amounts of 5M, the hybridisation started to melt at approximately 68°C with the fluorescence intensity decreasing at a slower rate. With excess non5-M the melting temperature was approximately 64°C.

Sensitivity of Detection of Methylated Cytosines

The molecular beacon method can readily detect methylation of the preferred target DNA when all 7 cytosine resides within the CpG dinucleotides are methylated (see Table 1). To determine the minimum number of cytosine residues that need to be methylated in order to alter the melt temperature of hybrids, preferred Myf-3 targets with variable numbers of methylated cytosine residues were reacted with beacon A in the test system. As shown in Table 2, five different modified preferred Myf-3 targets were examined. Modifications ranged from having one to five cytosine residues methylated. As illustrated in Fig. 2, beacon A hybrids with targets that have 2 or more cytosine residues dissociate at a significantly higher melt temperature compared to targets which have 1 or no methylated cytosine residues. Further work is required to establish test conditions which will allow distinction between the melt temperatures of unmethylated preferred target and a target which has one methylated cytosine residue.

Methylated Oligonucleotides

5-5M	5'- GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3' (M M M M M)
4-5M	5' - GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3' (M M M M)
3-5M	5' - GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3' (M M M)
2-5M	5' - GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3' (M M)
1-5M	5' - GCG GCG ACT CCG ACG CGT CCA GCC CGC GCT CC - 3' (M)

M=methylated cytosine residues.

NB. Reaction profiles shown in Fig. 2.

It should be understood that the foregoing description of the invention including the principles preferred embodiments and Examples cited above are illustrative of the invention and should not be regarded as being restrictive on its scope. Variations and modifications may be made to the invention by others without departing from the spirit of that which is described as the invention and it is expressly intended that all such variations and changes which fall within this ambit are embraced thereby.

Dated this FIRST day of FEBRUARY 1999

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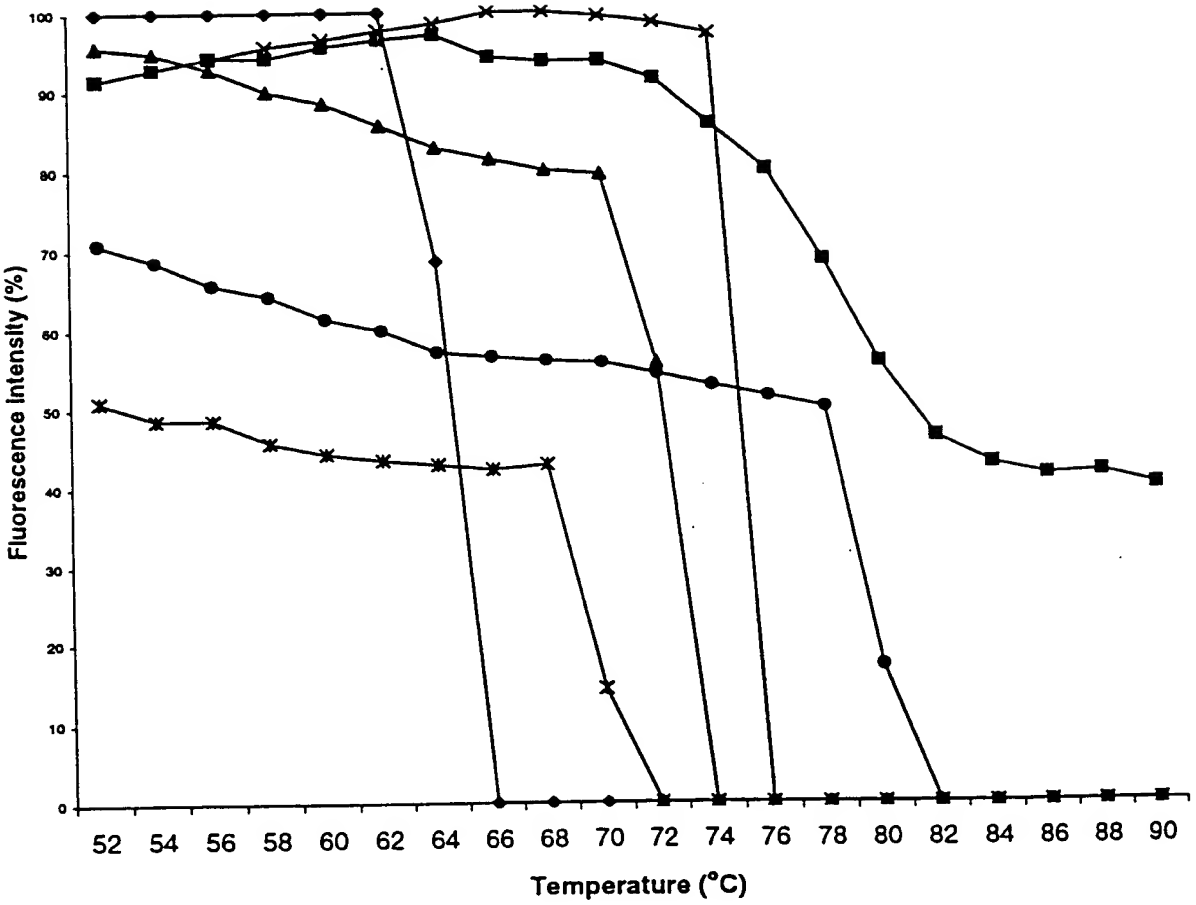
Fluorescence intensity (%)
100
90
80
70
60
50
40
30
20
10
0
52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90
Temperature (°C)

Fig. 1

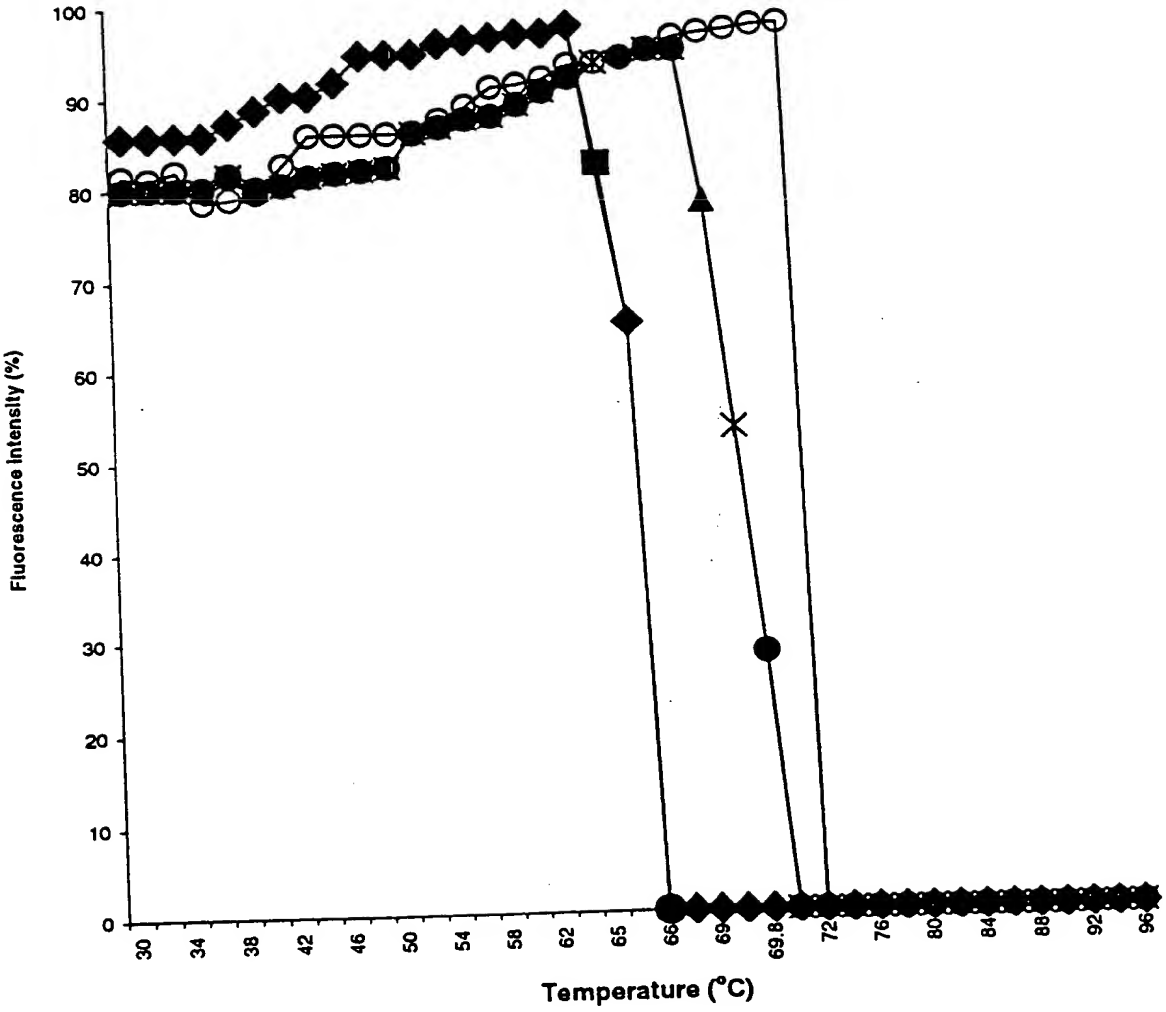
Fluorescence intensity (%)
100
90
80
70
60
50
40
30
20
10
0
30
34
38
42
46
50
54
58
62
65
66
69
69.8
72
76
80
84
88
92
96
Temperature (°C)

Fig. 2